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Review

The anti-metastatic micro-environment of the bone: Importance of osteocyte Cx43 hemichannels

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The anti-metastatic micro-environment of the bone: importance of osteocyte Cx43 hemichannels

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Invited Mini-review

Highlights

- Connexin 43 (Cx43) is an endogenous bone anti-metastatic factor for breast cancer cells present in osteocytes.
- Osteocyte Cx43 proteins function as mechanosensitive hemichannels that release anti-metastatic molecules like ATP.
- Bisphosphonate drugs, adjuvants applied in anti-breast cancer treatments, lead to osteocyte Cx43-hemichannel opening.
- Activation of osteocytic hemichannels by mechanical stimulation or bisphosphonate drugs suppress breast cancer metastasis in bones.
- Osteocyte Cx43 hemichannels are endogenous metastatic suppressors that are critical for the therapeutic response towards anti-metastasis drugs.

Abstract

Bone metastases of tumor cells are a common and life-threatening feature of a variety of late-stage cancers, including breast cancers. However, until now, much less has been known about the intrinsic anti-metastatic properties of the bones and how these could be exploited to prevent or treat bone metastases. Very recently, native Cx43 hemichannels present in osteocytes have been identified as important anti-metastatic signaling complexes by establishing high local extracellular ATP levels. Moreover, bisphosphonate drugs, applied as adjuvant therapies in the treatment of breast cancer patients and bone diseases, are known to display anti-metastatic properties. Now, it became clear that these compounds exert their effects by through osteocyte Cx43 hemichannels, thereby triggering their opening and promoting ATP release in the extracellular micro-environment. Hence, endogenous osteocyte Cx43 hemichannels emerge as important and promising therapeutic targets for the prevention and/or clinical treatment of bone-metastasized breast cancers.

Breast cancer metastases to the bone and clinical use of bisphosphonate drugs

Tumor cells often metastasize to different organs. This is a major life-threatening complication in many patients suffering from cancers, including breast cancer, at a late stage [1]. One of the major target organs for metastasis of cancer cells is the bone tissue. In the bone, osteocytes arise from osteoblasts trapped within the mineralized matrix. Osteocytes dynamically control bone density by impacting the function of bone-forming osteoblasts and bone-resorbing osteoclasts (reviewed in [2]). Different mechanisms for the occurrence of breast cancer metastases in the bone tissue have been proposed, including the production of osteoprotegerin by breast cancer cells and altered Runx2-dependent signaling in breast cancer cells [3, 4]. However, much less is known about the intrinsic anti-metastatic properties of the bone micro-environment. In addition to this, the relatively well-tolerated bisphosphonate drugs applied to treat bone loss and bone diseases, including cancer treatment-induced bone loss, also reduce the risk of bone metastasis [5, 6]. Bisphosphonates are associated with prolonged disease-free survival and with lower breast cancer incidence [7]. A meta-analysis revealed a significant benefit of having adjuvant bisphosphonate treatment in early breast cancer patients who were in post-menopause [8]. Very recently, a European panel has recommended the use of bisphosphonates as an adjuvant in the routine clinical practice for the treatment of early breast cancers, e.g. in post-menopause women [9, 10]. Yet, the therapeutic mode of action of bisphosphonates on the bone and the molecular targets participating in its physiological effects remained not fully understood.

Cx43 proteins function as gap junctions and hemichannels and are present in osteocytes

A major determinant for the physiological function of osteocytes in bones is the 43-kDa connexin protein (Cx43), a 4-transmembrane-domain protein with intracellular N-terminal, loop and C-terminal domains and two extracellular loops [11]. The Cx43 protein assembles into hexameric channels that can function as “free” hemichannels or “head-to-head”-docked gap junction channels [12, 13], establishing intercellular communication and coordination by the passage of low-molecular weight molecules (Mw < 1.5 kDa) [11]. Gap junctions allow the direct exchange of intracellular ions, like K^+, Na^+ and Ca^{2+}, signaling molecules, like IP_3, and molecules with biological functions, including short amino acid peptides and microRNAs. Hemichannels allow the exchange (release or entry) of low Mw molecules with the extracellular environment, establishing local, paracrine signaling networks and micro-environments through ATP, NAD^+, glutamate and prostaglandins [14]. For instance, Cx43 hemichannels can participate in the release of ATP, thereby eliciting purinergic receptor activation and Ca^{2+} signaling in neighboring cells, detectable as intercellular Ca^{2+} waves [15, 16]. Physiologically, in osteocytes, Cx43 hemichannels are mechanosensitive and become activated by fluid flow and shear stress [13]. Therapeutically, part of the bone-protective actions of bisphosphonate drugs could be attributed to its ability to trigger Cx43-hemichannel opening, thereby promoting cell survival and suppressing apoptosis in osteoblasts and osteocytes (critically reviewed in [17]). The presence of functional Cx43 hemichannels in osteoblasts and osteocytes was critical for the activation of cell survival signaling pathways, like Src and ERK, in response to bisphosphonate drugs and to protect against *in vivo* bone loss induced by glucocorticoids [18-20]. Very recently, the negative impact of glucocorticoids on osteocytes has been linked to a prominent decrease in Cx43-protein levels [21]. This is due to glucocorticoid-induced inhibition of Akt-mTORC1 signaling, which leads to an upregulation of autophagy [22], a major turn-over pathway for Cx43 gap junctions and hemichannels [22-25]. Indeed, independent studies previously showed that while high doses of glucocorticoids could induce apoptosis in osteocytes, low doses of glucocorticoids induced autophagy in osteocytes [26], which will negatively impact Cx43-protein levels.

Cx43 and its role in oncogenesis and cancer hall-marks

Cx43 in tumor cells

Cx43 expression has been linked to different tumor biological aspects for a variety of cancers, including breast cancers [27, 28]. This involves channel-dependent functions by promoting gap junctional and/or hemichannel-mediated intercellular communication and channel-independent functions by sequestering putative gene regulators and by providing Cx43-protein fragments that can translocate to the nucleus and regulate gene transcription [29]. Most insights about the role of Cx43 in the oncogenesis of cancer cells have been obtained on Cx43 channels present in tumor cells, where they impact different "hall-marks of cancer". In different cancer cell types, including glioma and breast cancers, Cx43 expression inhibits cell-cycle progression tumor growth, proliferation and migration, although Cx43 can also enhance cell migration and motility [29-31]. This dichotomous role for Cx43 is also reflected in its altered expression in different stages of tumor formation and spreading. Indeed, Cx43 expression is often lost during the early stages of the formation of primary breast tumors, while Cx43 expression is upregulated during metastasis and secondary tumor formation [28]. As such, Cx43 functions as a tumor suppressor in primary breast tumors, while it can act as either tumor suppressor or tumor promoter in advanced breast cancers at later stages dependent on the context [32].

Moreover, the effects of Cx43 expression on tumor cells might be dependent on its localization. For instance, in prostate LNCAP cancer cells, re-expressed Cx43 occurs at the cell membrane and increases tumor growth and invasion, while in PC-3 prostate cancer cells, re-expressed Cx43 occurs in the cytosol and prevents tumor growth and invasion. Also, in prostate cancer cell lines, Cx43 expression appeared to positively correlate with their increasing metastatic potential [33]. Knocking down Cx43 suppressed the metastatic properties of these cancer cells, while it did not impact their cell growth. Cx43 expression can also impact cell death and survival, processes often dysregulated in cancer cells. Cx43 gap junctions and hemichannels have been implicated in the spreading of cell death and survival factors, e.g. by facilitating the spreading of signaling molecules that become released upon mitochondrial outer membrane permeabilization, an important onset point for apoptosis [34, 35].

Cx43 in target tissues for hosting metastasized tumors

Besides the extensively studied role of Cx43 in the tumor cells, including breast cancers (recently thoroughly reviewed in [28]), Cx43 from host tissues also controls the eventual seeding and growth of the metastases and secondary tumors. Cx43 present in the host tissues promotes cancer cell migration and tissue invasion by forming hetero-cellular gap junctions between Cx43 from the tumor cells and Cx43 from the host tissue [30]. Via these hetero-cellular gap junctions, glioma cells can transfer a specific subset of microRNAs to astrocytes, like miR-5096, which contributed to the pro-invasive effect [36]. The pro-invasive role of Cx43 has recently been observed *in vivo* in the peri-tumoral region between glioma cells and astrocytes [37]. Absence of Cx43 in the tumors or in the astrocytic host environment attenuates invasion in the astrocyte environment. Very recently, further mechanistic insights on the interplay between metastasized tumor cells and astrocytes have been obtained [38]. New Cx43 gap junctions are formed between tumor cells and astrocytes through protocadherin 7, which is expressed in astrocytes and several cancer cells, including the ones originating from breast and lung tissues. These hetero-cellular Cx43 gap junctions between cancer cells and astrocytes provide an exchange pathway for second messenger cyclic GMP-AMP (cGAMP) signaling molecules from cancer cells towards astrocytes. In astrocytes, cGAMP initiate "stimulator of interferon genes" (STING) signaling, an innate immune response pathway activated upon double-standed DNA appearance in the cytosol [39]. STING activation results in the production and release of inflammatory cytokines

interferon-α and tumor necrosis factor from astrocytes. As a consequence, tumor cells are influenced by these paracrine factors, activating STAT1 and NK-κB pathways, which favor tumor growth and promote chemoresistance [38]. However, besides the pro-metastatic roles of native Cx43, like in astrocytes, Cx43 expression in other host tissues like the lungs can also counteract tumor metastasis and cancer growth in these tissues, as lack of proper Cx43 expression in the host organism facilitated the occurrence of mammary gland tumor metastasis to the lungs [40].

However, until now, the impact of Cx43 channels on tumor metastasis in the bone and their role in the anti-metastatic properties of bisphosphonate drugs were poorly understood. In particular, most studies describing a role for Cx43 in metastasis and invasion reported its contribution as gap junctional channels, but their role as hemichannels in these processes remained largely unexplored. This was largely due to the fact that Cx43 knockout approaches will obviously impact both Cx43 gap junctions and hemichannels, while selective Cx43 gap junction inhibitors remained not without side effects. Yet, recent insights in the distinct regulation of Cx43 gap junctions *versus* hemichannels by intramolecular loop/tail interactions have opened novel opportunities to design mutant channels and peptide tools that could abolish gap junctional channel activity while retaining hemichannel activity and *vice versa* [14, 41-46]. These insights, tools and experimental approaches now provide unprecedented opportunities to unravel (patho)physiological functions of Cx43 as gap junctional channels *versus* hemichannels [43].

The role of osteocyte Cx43 hemichannels in establishing an endogenous anti-metastatic bone micro-environment and in the therapeutic response towards anti-metastatic drugs

Recent studies from the Jiang team revealed that endogenous Cx43 hemichannels present in osteocytes function as novel anti-neoplastic factors preventing bone metastasis of breast cancer cells [47, 48] (see Fig. 1 for the model). Medium collected from osteocytes exposed to bisphosphonate drugs, like alendronate, inhibited the migration of breast cancer cells and anchorage-independent growth. This migration was dependent on ATP released from osteocytes and on purinergic $P2X_7$ receptor-mediated signaling in the breast cancer cells. Indeed, ATP-degrading enzymes or $P2X_7$ antagonist alleviated the inhibition of breast cancer cell migration, while $P2X_7$ agonists by themselves were able to inhibit breast cancer cell migration. ATP by itself had a biphasic effect on the migration by breast cancer cells with low concentration causing inhibition of migration while high concentration promoting migration [47]. This effect could be attributed to the metabolism of ATP, since non-hydrolysable ATP only inhibited migration, while adenosine, a metabolic product of ATP, only promoted migration. In follow-up work, the authors showed that bisphosphonate drugs inhibit breast cancer cell migration and anchorage-independent tumor growth. These anti-metastatic properties of bisphosphonates were attributed to their ability to trigger ATP release from osteocytes by opening Cx43 hemichannels, since an anti-Cx43 antibody directed against the extracellular loop that inhibits Cx43-hemichannel opening abolished the anti-migration effect [48]. Interestingly, osteoblast cells expressing Cx43 did not release ATP in response to bisphosphonate drugs and the medium collected of these cells did not impact breast cancer cell migration, indicating a critical role for Cx43 hemichannels present in osteocytes. Also, not only alendronate but also zoledronic acid, an FDA-approved drug for the treatment of bone metastases, could trigger Cx43-hemichannel opening from osteocytes and suppress cell growth and migration of breast cancer cells, suggesting that activation of Cx43 hemichannels may be a common feature of different bisphosphonate drugs. Moreover, not only pharmacological tools but also physiological conditions like mechanical stimulation of osteocytes by fluid flow shear stress also triggered Cx43-hemichannel-mediated ATP release and the medium of osteocytes exposed to a physiological form of mechanical stimulation was able to inhibit breast cancer

cell migration. This indicates that also physiological opening of Cx43 hemichannels present in osteocytes can prevent metastasis. This is underpinned by *in vivo* experiments using an osteocyte-specific Cx43-knockout mouse model. In comparison to wild-type mouse, breast cancer cells grew much faster in the osteocyte-specific Cx43 knockout mouse model. Since the Cx43 protein serves as a building block for both gap junctions and hemichannels, a Cx43 knockout cannot identify whether the anti-metastatic properties are due to Cx43's function as gap junctions or hemichannels. Therefore, the authors elegantly used two mouse models, one in which osteocytes were lacking functional Cx43 gap junctions but were expressing functional Cx43 hemichannels ($Cx43^{R76W}$) and one in which osteocytes were lacking functional Cx43 gap junctions and hemichannels ($Cx43^{\Delta 130-136}$) (see Fig. 2 for the Cx43 mutants used and their functional outcome). Importantly, tumor growth in the bones was significantly accelerated in the $Cx43^{\Delta 130-136}$ mouse model compared to the wild-type mice or $Cx43^{R76W}$ mice. Furthermore, treatment of the mice with bisphosphonates drugs suppressed tumor growth in the bones of wild-type mice or $Cx43^{R76W}$ mice, but its anti-tumor properties were abolished in mice expressing $Cx43^{\Delta 130-136}$. Of note, also mechanical loading, a physiological stimulus for the opening of osteocytic Cx43 hemichannels, inhibited the migration of breast cancer cells. This is important, given the beneficial effects of physical exercise, which likely is associated with increased mechanical stimulation of osteocytes due to fluid flow, towards reduced tumor growth and metastasis. Thus, these studies now identified (i) Cx43 hemichannels present in osteocytes as novel tumor suppressors counteracting bone metastasis and tumor growth and (ii) the pharmacological targets of bisphosphonate drugs used in the clinic to treat bone metastasis. The mechanism of action of these drugs involves the opening of Cx43 hemichannels from osteocytes. Thus, this study further underpins the emerging physiological functions of Cx43 hemichannels as ATP-release pathway in a variety of cell systems, tissues and organs. Here, Cx43 hemichannels in the bone are shown to possess a "self-protective" action that counteracts the metastatic behavior of breast cancer cells, avoiding bone tissue colonization by these cancer cells.

Remaining questions and future directions

However, this study also raises a number of fundamental questions and challenges for the therapeutic applications of these concepts for the benefit of human health in anti-cancer treatments. First of all, it will be interesting to establish whether the anti-metastatic potential of osteocyte Cx43 hemichannels can be expanded to other tumors besides breast cancers, as obviously many tumors can metastasize towards the bones. Second, other sites of metastasis include the lungs and the brain, but the contribution and/or potential of hemichannels established by Cx43 (or other Cx isoforms) to counteract metastases in these tissues remains unknown. Also, at the level of the mechanism of action of bisphosphonate drugs towards (specific?) activation of osteocytic Cx43 hemichannels further work is needed. For instance, it is not clear how bisphosphonate drugs result in the opening of Cx43 hemichannels from osteocytes. The signaling pathways involved ought to be elucidated. Interestingly, it appears that the bisphosphonate drugs are selectively acting on Cx43 hemichannels present in osteocytes, since osteoblasts expressing Cx43 hemichannels do not release ATP upon bisphosphonate drug exposure, although other studies convincingly challenge this concept (reviewed in [17]). Thus, the difference between osteoblasts and osteocytes in responsiveness to bisphosphonate drugs will be an interesting avenue to explore. Importantly, work from Morelli and team using Cx43-expressing and Cx43-deficient cells argued against a direct binding of alendronate to Cx43 hemichannels and also excluded Cx43 hemichannels as an uptake route for bisphosphonate drugs in osteocytes/osteoblasts [49]. Also, in this work, the anti-apoptotic effects of bisphosphonate drugs were dependent on Cx43 hemichannels, while their proliferative effects on osteoblasts were independent of the presence of

Cx43 hemichannels [49].
In addition, the impact of bisphosphonate drugs on Cx43 hemichannels present in other cell types or tissues, including astrocytes or cardiomyocytes, ought to be scrutinized, as this could lead to serious adverse side-effects. Furthermore, further long-term studies on osteocytes are needed to show whether bisphosphonate could impact the viability of osteocytes, since excessive Cx43-hemichannel opening could lead to a collapse of ionic, metabolic and energetic gradients essential for cell survival. Also, at the level of the breast cancer cells, the anti- and pro-neoplastic signaling pathways and processes activated in response to ATP-mediated $P2X_7$ receptor stimulation and adenosine-mediated adenosine receptor stimulation, respectively, ought to be further characterized.

At the therapeutic level, it will be important to determine whether *in vivo* treatments with bisphosphonate drugs are effective in inhibiting tumor growth in bones, definitely in long-term settings. Furthermore, ATP released from osteocytes was shown to display biphasic effects on breast cancer cell migration due to the stimulatory effects of adenosine, a metabolic product of ATP, on this process [50]. As bisphosphonate drugs exert part of their anti-metastatic effects by opening osteocyte Cx43 hemichannels and raising extracellular ATP in the tumor micro-environment, the appearance of adenosine in the tumor micro-environment, which promotes tumor growth, migration and metastasis [50] and which leads to impaired immune-surveillance [51], may hamper their long-term therapeutic applicability. Although different processes contribute to the accumulation of adenosine in the tumor micro-environment, the presence of ectonucleotidases like CD39 and CD73 at the surface of breast cancer cells or secreted via exosomes is the main mechanism for producing extracellular adenosine by catalyzing the following conversions: ATP $\rightarrow$ ADP $\rightarrow$ AMP $\rightarrow$ adenosine [52]. Hence, combination strategies with inhibitors of the ectonucleotidases CD39 and CD73 or antagonists of adenosine-mediated signaling pathways could overcome the adverse effects of the ATP-metabolic product adenosine. In addition, these CD73 and A_{2A} inhibitors will also help to counteract tumor metastases by boosting host anti-tumor immune responses. For instance, blockade of A_{2A} receptors suppressed metastases by promoting the maturation and function of natural killer cells [53]. Also, an inhibitory anti-CD73 monoclonal antibody inhibits tumor growth and metastases in immune-competent but not in immune-deficient mice [54].

Another important aspect to consider is the endogenous Cx43 protein-levels in the bone of cancer patients. Since bisphosphonate drugs exert their function by opening Cx43 hemichannels in osteocytes, the Cx43-expression levels and its occurrence as hemichannels in osteocytes will be crucial for the therapeutic effect of these drugs. In particular, it should be examined whether Cx43 levels in osteocytes remain sufficiently high in cancer patients to establish sufficient Cx43 hemichannels and thus to sufficiently raise ATP levels in the osteocyte/tumor micro-environment. Thus, tools that favor Cx43-hemichannel opening in response to activating stimuli may boost the anti-metastatic properties of bisphosphonate drugs. Such tools have recently become available based on insights in the molecular determinants underlying Cx43-hemichannel activation. It was discovered that intramolecular interactions between in the cytoplasmic loop of Cx43 and the last 10 amino acids of the Cx43 C-terminal tail not only control Cx43 gap junction activity but also Cx43-hemichannel activity. Strikingly, while such interactions close Cx43 gap junctions, they are critical to render Cx43 hemichannels in a "ready to open" state [44]. Thus, cell-permeable peptides representing parts of the cytoplasmic loop, like TAT-L2, Gap19 or TAT-Gap19, inhibit Cx43 hemichannel opening in a variety of cellular systems in response to physiological stimuli like moderate $[Ca^{2+}]_{cyt}$ increases [45, 46], extracellular Ca^{2+} buffer [45, 46, 55, 56], mechanical stimulation [45] or depolarization [44], while favoring Cx43 gap junctional channel opening [57]. Moreover, these peptide tools can also limit excessive Cx43 hemichannel openings in response to pathophysiological stimuli like ischemia reperfusion cardiac systems [46]. This suppresses cell damage to these tissues, thereby maintaining their physiological functions [41, 42, 58].

In contrast, cell-permeable peptides representing the last 10 amino acids, like TAT-CT10, can prevent the closure of Cx43 hemichannels in conditions that activate the actomyosin contractility like high $[Ca^{2+}]_{cyt}$ [45, 59]. As such, TAT-CT10 peptide may promote the effectiveness of bisphosphonate drugs by favoring the opening of endogenous osteocyte Cx43 hemichannels. The ability of these tools to promote endogenous Cx43 hemichannel activity has recently been established in nodose ganglion sensory neurons, thereby increasing their excitability [60]. Interestingly, a related peptide, ACT1, an antennapedia-coupled CT10 peptide, which promotes gap junctional assembly by interfering with Cx43/ZO-1 complexes and sustains intercellular coupling [61], enhanced the activity of tamoxifen and lapatinib in breast cancers [62]. Thus, beyond their beneficial role in wound healing [63-65] and maintaining gap junction coupling in cardiac injury paradigms [63], CT10-based tools might be very effective adjuvant molecules in anti-cancer strategies in two ways: (i) by promoting Cx43 gap junctional coupling between breast cancer cells favoring sensitivity to targeted inhibitors, e.g. through `bystander` effects [34, 35] and (ii) by enhancing endogenous Cx43-hemichannel activity from osteocytes boosting the effects of bisphosphonate drugs to establish an ATP-rich, anti-metastatic micro-environment.

Finally, Cx43-hemichannel opening has been associated with a plethora of pathophysiological conditions, including ischemia/reperfusion in the brain and heart, inflammatory conditions, neurodegenerative conditions like amyloid beta accumulation [58, 66, 67]. As such, inhibition of Cx43 hemichannel opening has been proposed as a promising therapeutic application for these conditions [46, 58]. These findings together with advances in understanding the mechanisms that control Cx43-hemichannel activity have spurred the development of selective Cx43 hemichannel inhibitors that do not negatively impact Cx43 gap junction activity [41, 42, 44, 45]. Yet, the timeframe of treatments with Cx43-hemichannel inhibitors will be an important aspect to consider. Indeed, given the inherent tumor suppressive and anti-metastatic role of osteocytic Cx43 hemichannels, long-term treatments with Cx43-hemichannel inhibitors might adversely impact the risk for developing cancer metastasis to the bone, e.g. in patients with breast cancer. However, the application of Cx43-hemichannel inhibitors to limit cell damage in response to ischemia/reperfusion is anticipated to be a relatively short-term intervention, thus likely avoiding a negative impact on the anti-metastatic properties of osteocytic Cx43 hemichannels.

Conclusions

Overall, over the years, it has become clear that Cx43-hemichannel openings are not only associated with pathological conditions, but are also involved in important physiological signaling processes by releasing molecules like ATP and glutamate. Now, for the first time, Cx43-hemichannel opening is associated with antagonizing a pathological condition, namely cancer cell metastasis in the bones, by mediating local ATP release from osteocytes in response to physiological stimuli (mechanical stimulation) and therapeutics (bisphosphonates). Hence, osteocytic Cx43 hemichannels act as native and endogenous suppressors of breast cancer metastases in the bone and serve a crucial role in the therapeutic response towards bisphosphonate-based drugs, already clinically used as chemotherapy adjuvants for treating bone metastases. These findings open exciting avenues for further fundamental and translational research, including the therapeutic application of these concepts in the clinical treatment of breast cancer patients to prevent or cure bone metastasis.

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Legend

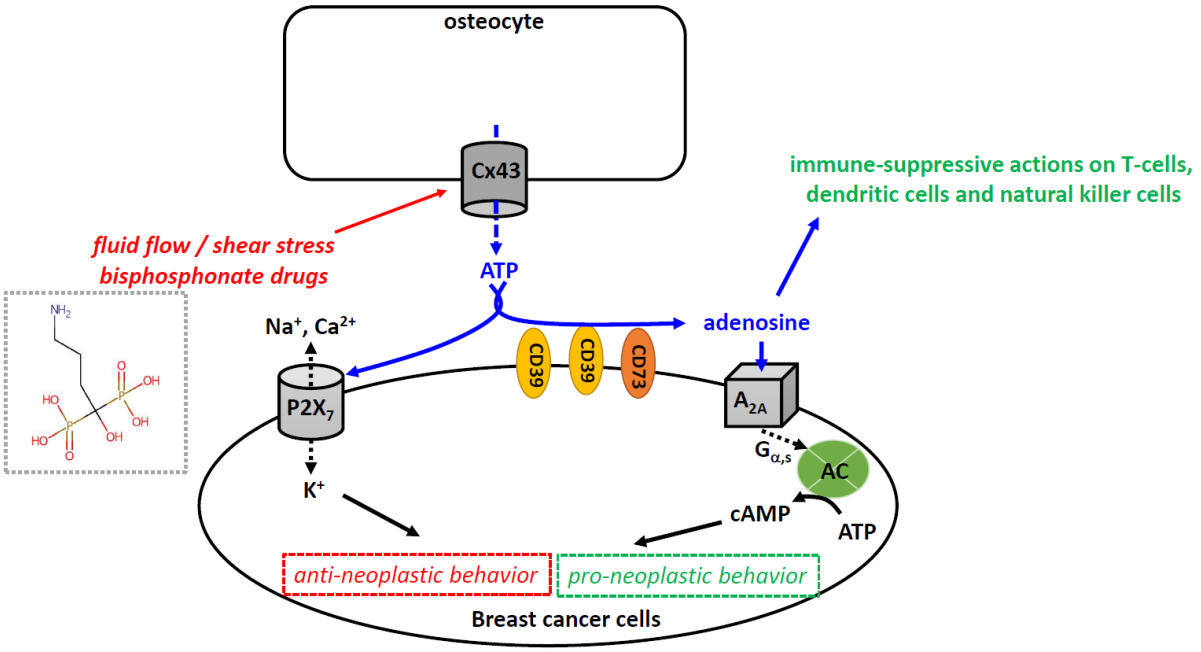
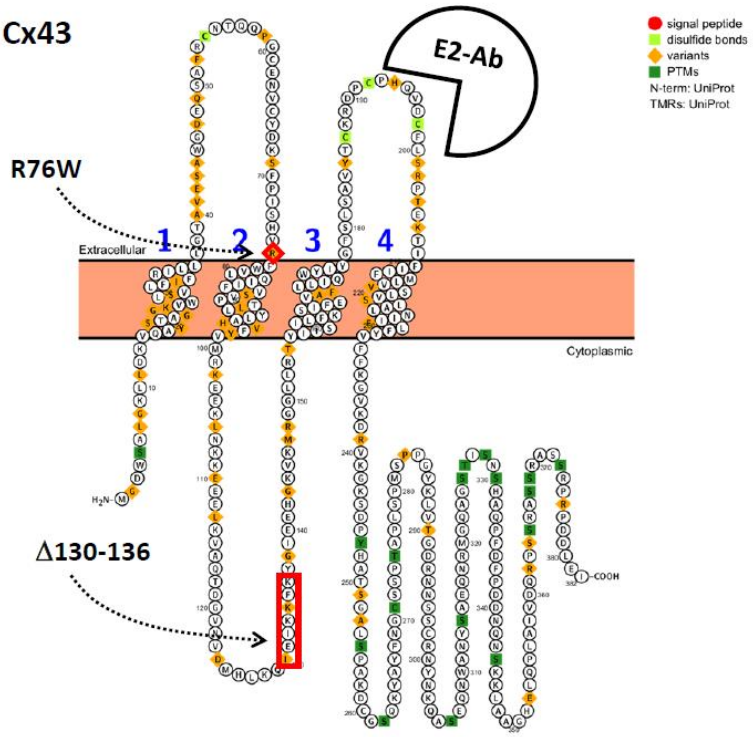


Figure 1. Osteocytic Cx43 hemichannels as anti-metastatic factors in the bone. Cx43 is important for the function and viability of osteocytes by acting as mechanosensitive hemichannels mediating the release of signaling molecules, including ATP. Recent work from the team of Jiang revealed that Cx43 hemichannels function as endogenous suppressors of breast cancer cell metastasis in the bone and are crucial for the therapeutic response towards bisphosphonate drugs, compounds used in the clinic as adjuvant for the treatment of early breast cancers. Opening of osteocyte hemichannels, in response to physiological stimuli like mechanical loading or to pharmacological stimuli like bisphosphonate drugs (structure of alendronate is shown; taken from http://images.ddccdn.com/img/mol/DB00630.mol.jpg), result in the release of ATP, a signaling molecule exerting anti-neoplastic function on breast cancer cells by acting on the purinergic $P2X_7$ receptors. Of importance, adenosine a metabolic product of ATP exert pro-neoplastic action on breast cancer cells by acting on the adenosine receptor A_{2A}. Thus, strategies that counteract ATP degradation (e.g. by inhibiting the ectonucleotidases CD39 and CD73) and thus prevent adenosine accumulation in the tumor micro-environment may be needed to further to maximize the therapeutic effects of bisphosphonate drugs in the prevention and/or treatment of breast cancer metastasis.

A



B

osteocyte	gap junctions?	hemichannels?	anti-metastatic?
Cx43	yes	yes	yes
$Cx43^{R76W}$	no	yes	yes
$Cx43^{\Delta 130-136}$	no	no	no
Cx43 + E2-Ab	yes	no	no

Figure 2. Overview of the osteocytic Cx43 mutants and Cx43-targeting tools like the E2 antibody used by Jiang and co-workers [48] and their functional outcome in terms of gap junctions, hemichannels and anti-metastatic potential in response to bisphosphonate drugs. *A*, A schematic illustration of the human Cx43 protein sequence (Protein Accession Number used: P17302) using the Protter protein-visualization tool (http://wlab.ethz.ch/protter/start/; [68]), which represents one building block of the hexameric hemichannel and the head-to-head-docked hemichannel that establish a gap junction channel. The location of the relevant mutations and deletions used by Jiang and co-workers are highlighted in a red box, i.e. $Cx43^{R76W}$ and $Cx43^{\Delta 130-136}$. The E2 antibody (E2-Ab), targeting the second extracellular loop, is also depicted. *B*, A table representing the functional outcome and impact on the anti-metastatic properties of osteocytes and bone micro-environment of wild-type Cx43, Cx43 mutants, selectively expressed in osteocytes, and the E2 antibody targeting Cx43. Osteocytes expressing Cx43, which establish functional gap junctions and hemichannels, suppress breast cancer metastasis to the bone in response to physiological stimuli and bisphosphonate anti-metastatic drugs. Osteocytes expressing $Cx43^{R76W}$, which can form functional hemichannels but not functional gap junctions, remains capable of counteracting bone metastases. This excludes that Cx43 gap junctions are required for the anti-metastatic properties of bisphosphonates. Osteocytes expressing $Cx43^{\Delta 130-136}$, which can neither form functional gap junctions nor functional hemichannels, lost their ability to suppress bone metastases. Selective inhibition of Cx43 hemichannels using the E2 antibody (E2-Ab), thereby maintaining gap junction function, is able to neutralize the anti-metastatic properties of osteocytes upon exposure to bisphosphonate drugs in *in vitro* experiments